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(54) Title: MODIFIED DNA VIRUS VECTORS AND USES THEREFOR

(57) Abstract

Methods and compositions are described for production of desired peptides or proteins *in vitro* or *in vivo*. A system for rapidly inserting exogenous DNA encoding a selected protein or peptide into a DNA virus is provided. One embodiment of the invention includes a modified HSV-1 virus, which comprises at least one restriction endonuclease site foreign to wild-type HSV and a universal cloning cassette containing the exogenous DNA flanked by the foreign restriction enzyme site and a restriction enzyme site native to the HSV-1 virus.

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MODIFIED DNA VIRUS VECTORS AND USES THEREFOR

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Field of the Invention

The present invention relates generally to methods and compositions for use in expressing desired heterologous peptides and proteins in a modified virus vector.

Background of the Invention

Herpes Simplex Virus-1 (HSV-1) is an enveloped DNA animal virus, a member of the family Herpesviridae, which typically causes skin lesions in humans and animals, and has a large and complex DNA genome of about 150kb. After initial replication at the site of infection, HSV-1 establishes latent infection in the nervous system, e.g., the ganglia, from which it can be reactivated by stimuli, such as colds, stress and sunlight, among others. In the latent period, none of the HSV-1 genes are detectable except the latency associated transcript (LAT) gene, unless reactivation occurs [T.J. Hill, "Herpes simplex virus latency", in B. Roizman (ed), The Herpes Viruses, Plenum Publishing Corp., New York, pp 175-240 (1985); V. R. Baichwal et al, Cell, 52:787-789 (1988); J. G. Stevens, Microbiol. Rev., 53:318-332 (1989); and N. W. Fraser et al, Virology, 191:1-8 (1992)].

The LATs, including the LAT promoters, of HSV-1 have been thoroughly studied. The LAT sequences have been mapped to one small region of the viral genome - the repeat long region [A. M. Deatly et al, Proc. Natl. Acad. Sci. USA, 84:3204-3208 (1987); D. L. Rock et al, J.

Virol., 61:3820-3826 (1987); J. G. Spivack et al, J. Virol., 61:3841-3847 (1987); J. G. Stevens et al, Science, 235:1056-1059 (1987); and E. K. Wagner et al, J. Virol., 62:4577-4585 (1988)]. Further, their promoter has been mapped to a specific TATAA box within the repeat long region of the viral genome. The LAT transcripts accumulate in large amounts in peripheral nervous system (PNS) and central nervous system (CNS) tissues [A. M. Deatly et al, J. Virol., 62:749-756 (1988)].

HSV-1 has been employed as a vector in recombinant expression systems. Because the LAT gene product has been seen to accumulate to high levels in the neuronal cells of latently infected animals and humans, it has been used to express foreign genes. A vector system from HSV-1 was designed in which the β-glucuronidase (GUSB) gene has been inserted approximately 400 nucleotides downstream of the LAT TATA box; thus, its expression is under control of the LAT promoter. Long term expression was achieved in both PNS and CNS tissue following transfer into GUSB- mice [J. H. Wolfe et al, Nature Gen., 1:379-384 (1992)].

Other groups have constructed viruses containing the β-globin gene inserted 26 bases downstream of the TATA box of the LAT promoter [A. T. Dobson et al, J. Virol., 63:3844-3851 (1989)], and the β-galactosidase gene inserted 823 bp downstream of LAT TATA box consensus sequences [D. Y. Ho et al, Proc. Natl. Acad. Sci. USA, 86:7596-7600 (1989)]. All three recombinant viruses have been able to express the foreign genes under regulatory control of the LAT promoter in the neurons of sensory ganglia of latently infected mice once latency is established, but are silent or expressed at very low levels in tissue culture and during the acute infection period. Expression in the central nervous system has not be demonstrated.

A common method for inserting foreign genes into large genomes (e.g. ≥ 150,000 bases) at a position of choice and preparing these vectors of the prior art is homologous recombination [B. Roizman et al, "Herpesviruses and their replication", B.N. Fields (ed), in Virology, Raven Press, Ltd., New York, pp 497-526 (1985)]. This procedure is very time consuming, results in a low yield of recombinants, and requires several rounds of plaque purification which can take many months for each change in the genome. Furthermore, the recombination event can result in unintended rearrangement of the inserted DNA sequence. Therefore, as a means for developing vectors requiring a number of changes in the genome, repeated usage of homologous recombination is not preferred.

Another method of making recombinant vectors of HSV-1 involves the use of amplicons, which are defective viral particles containing sufficient genetic information to allow DNA replication and packaging in the presence of a helper virus. This method has been used to study the packaging sequences and origins of replication of HSV [R. R. Spaete et al, Cell, 30:295-304 (1982)]. See also, International Application Publication No. WO90/09441 (published August 23, 1990). HSV-1 amplicons have been suggested to reduce the time required to construct vectors containing altered genes and allow the relatively simple preparation of large numbers of related gene constructs for studying gene expression in the nervous system *in vivo*. However, the inventors found that these amplicons do not package efficiently when foreign DNA is inserted. Further, amplicons are contaminated with helper virus, which can cause disease.

Another method for the introduction of foreign genes into specific sites within the viral genome is the Cre-lox recombination system derived from phage P1. This

system consists of a 34-bp lox P DNA sequence and a 38-KDa Cre recombinase protein [P. J. Gage et al, J. Virol., 66:5509-5515 (1992); and M. K. Sauer et al, Mol. Cell Biol., 8:1011-1018 (1988)]. It is a more efficient system than homologous recombination, with related recombination frequencies up to 15%, but still requires several rounds of plaque purification which can take months.

Another HSV expression system was described in Rixon and McLauchlan, J. Gen. Virol., 71:2931-2939 (1990), in which wild type HSV was mutated by eliminating its naturally occurring XbaI restriction sites, and a unique XbaI site was inserted downstream of the immediate early promoter to permit insertion of a foreign gene flanked by XbaI sites. However, mutation of HSV to remove XbaI sites damaged the replication ability of this virus. Additionally, this virus vector is active in infected neuronal cells *in vivo* only during the acute phase of HSV-1 replication.

See also, European Patent Publication No. 453,242 (October 23, 1991) which describes HSV mutants containing deletions in genes for viral replication, particularly the immediate early genes. Another mutant HSV-1 is described in International Application Publication No. WO91/02788 (March 7, 1991). These vectors have a substantial portion of the Vmw65 protein removed to prevent *in vivo* viral replication.

Other viruses have potential use as viral vectors for the production of heterologous peptides and proteins *in vitro* or *in vivo*, such as other *Herpesviridae* species, e.g., Epstein Barr virus, cytomegalovirus and varicella zoster virus.

There exists a need in the art for a vector system which will surmount the above-stated problems with respect to viral vectors and will offer a substantially

faster method of generating recombinant vector viruses than the prior art, thus enabling the production of heterologous peptides and proteins in viral vectors.

Summary of the Invention

In one aspect, the present invention provides a modified DNA virus useful in a method for producing a selected peptide or protein. The virus is produced by inserting by homologous recombination one, or two, unique restriction enzyme site(s) foreign to the unmodified virus at a selected locus in the genome. The virus is susceptible to further modification by insertion of various exogenous DNA sequences by ligation at the unique restriction site(s). Where two unique, but different, sites are present in the modified virus, the ligated fragment may be inserted directionally.

In another aspect, the present invention provides a further modified virus as described above further comprising a reporter gene ligated into the unique restriction sites of the virus in a manner so as to permit the resulting modified virus to express the product encoded by the reporter gene.

In another aspect, the present invention provides a method for expressing an exogenous DNA sequence encoding a selected peptide or protein in a selected DNA virus. The method uses the DNA viruses modified as described above. According to the method, the virus containing the unique restriction site(s) is digested with restriction enzymes specific for said site(s) and a first plasmid cassette is inserted (ligated) into the digestion sites. This first cassette comprises a universal cloning sequence flanked by two restriction enzyme sites which are the same as the unique restriction enzyme site(s), at least one of which is foreign to the viral genome. Desirably, a reporter gene

is inserted into the universal cloning site using conventional ligation techniques. When the modified virus is grown in tissue culture, viral plaques which are visually distinct due to expression of the reporter gene are generated. Where lacZ is the reporter gene, the viral plaques are blue. The modified virus is digested with the unique restriction enzymes to remove the reporter gene. Into the digestion site a second plasmid cassette is inserted which comprises a DNA sequence encoding a selected protein or peptide flanked by the unique restriction site(s). Virus is then grown in tissue culture. The viruses capable of producing the protein or peptide encoded by the sequence of the second cassette are distinguished from viruses incapable of such production because of the difference between the viral plaques.

In still another aspect, the invention provides an expression system for efficiently introducing a selected exogenous gene into a selected DNA virus, such as a member of the family Herpesviridae, e.g., Herpes simplex virus (HSV), which system comprises a modified virus as described above and a recombinant DNA sequence component, namely a DNA sequence or "cassette" which comprises a universal cloning site sequence flanked at each end by the same unique restriction enzyme recognition sites of the modified virus component.

In still another aspect, the present invention provides a method for efficiently introducing a selected exogenous gene into a DNA virus under the control of a promoter which controls expression thereof by employing the components described above. This method is described in more detail below.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Figures

Fig. 1 illustrates the construction of plasmid pBPX-4Z4 which contains the PacI sites and the HSV-1 flanking LAT sequences.

Fig. 2 illustrates the engineering of the PacI recombinant virus HFEM/pICP6-lacZ by homologous recombination.

Fig. 3 provides a map of the HFEM genome and BamHI B and E region of the recombinant virus HFEM/pICP6-lacZ.

Fig. 4 illustrates *in vitro* ligation and transfection and the lacZ blue/white selection scheme.

Fig. 5 provides a map of the HFEM genome and BamHI B and E region of recombinant virus HFEM/pLAT-lacZ.

Detailed Description of the Invention

The present invention provides a simple, rapid, and efficient method, and the components thereof, to introduce exogenous DNA sequences encoding desired protein or peptide genes into the genome of a DNA virus, preferably a large virus such as a member of the family *Herpesviridae*, resulting in modified viruses useful in the production of exogenous or heterologous peptides and proteins of interest.

I. Definitions

A "reporter gene" is a gene sequence which upon expression results in the production of a protein whose presence or activity can be easily monitored. Such a gene may also be a selectable marker gene.

The "lacZ gene" is a reporter gene which encodes the β-galactosidase activity of *E. coli*. In the presence of its substrate, X-gal, the lacZ protein turns blue.

A "selectable marker gene" is a gene sequence capable of expressing a protein whose presence permits the selective propagation of a cell containing it.

A "vector" is any genetic element, e.g., a plasmid, chromosome, virus, etc., that is capable of replication under its own control, to which another polynucleotide segment is attached, such as to bring about the replication and/or expression of the attached segment.

A "promoter" is a region of DNA capable of binding RNA polymerase and directing the enzyme to the correct transcriptional start site. In one preferred embodiment of the present invention, the promoter sequence is one that is active during the herpes virus latent period.

A "universal cloning sequence" is a polynucleotide sequence which comprises one or more sequential or overlapping restriction endonuclease enzyme cleavage sites and thus provides a suitable location for ready deletion and insertion of an exogenous gene or other DNA sequence flanked by complementary restriction sites.

An "exogenous gene" is any gene not naturally present in the genome into which it is inserted or attached.

A "therapeutically desirable protein", as defined herein, is a protein which has been associated with a neural condition or symptoms thereof and which, when internally administered, is useful in alleviating the condition or symptom(s).

A "neural condition" is a physiological state in which a disease or imbalance of the central or peripheral nervous systems are involved or affected.

II. The Invention

According to this invention, a system which enables the expression of a desired protein or peptide in a DNA virus is designed for expression of the protein or peptide *in vitro* or, if suitable, *in vivo*. The novel expression system utilizes both a modified viral vector containing a unique restriction site as described in detail below and a second component, which is a DNA sequence or "cassette" which contains a universal cloning sequence flanked by selected endonuclease enzyme restriction sites which are unique and at least one of which is foreign to the selected wild-type DNA virus vector. An exogenous gene and optional promoter may be inserted into the cassette by conventional recombinant technology. This component is itself capable of being inserted into the viral vector component.

This invention involves manipulating a selected wild-type virus, such as a member of the family *Herpesviridae*, which includes, Herpes Simplex virus (HSV), Epstein Barr virus (EBV), Cytomegalovirus (CMV) and Varicella zoster virus (VZV), among others. For simplicity in description, HSV-1 is discussed as the exemplary, preferred virus for use in this invention. The choice of HSV-1 strains to be used for engineering recombinant virus vectors of this invention may be made by one of skill in the art. For safety, both *in vitro* and *in vivo*, it is preferable to employ strains of HSV-1 with reduced neurovirulence and increased neuroinvasiveness, as "backbones" for engineering the virus-derived vectors. Such viral strains also increase distribution of the exogenous gene if the vector is to be employed *in vivo*.

It should be understood that wherever in this description HSV is referred to, the procedures may also be applied to other DNA viruses including those of the

Herpesviridae family identified above. For example, by applying the teachings described herein to other DNA viruses such as VZV, EBV, and CMV, vectors such as exemplified herein for HSV can be rapidly produced with these viruses and selected proteins and peptides expressed thereby. Still other DNA viruses with large genomes, e.g., about 150 kb or greater, may be used in this invention.

According to this invention, a selected wild-type virus is modified by the introduction by homologous recombination of one or two unique restriction endonuclease enzyme cleavage sites which are foreign to that wild-type virus. Where a single foreign enzyme cleavage site is inserted into the viral genome, it is desirably inserted within 4000 - 5000 base pairs of a restriction enzyme cleavage site which is endogenous (native) and unique to the wild-type virus. For example, wild-type HSV-1, strain HFEM, is characterized by a single SwaI site. Thus, in a preferred embodiment of the present invention, a single exogenous enzyme cleavage site, e.g. PacI, is engineered into the genome of HSV-1 within 4 - 5 kilobases of the SwaI site. This permits directional insertion of a foreign DNA sequence, as discussed below.

Alternatively, two foreign enzyme cleavage sites can be inserted into the herpes virus genome. These cleavage sites may be the same, if directional insertion of a foreign gene is not critical. For example, if desired, two PacI sites could be engineered into EBV or HSV, as the PacI site is foreign to both these wild-type viruses. However, preferably, the enzyme cleavage sites are different from one another and are both foreign to the herpes virus genome. For example, if the target viral genome is CMV, any combination of two sites not found in the wild-type CMV, e.g. PacI, SwaI and

PmeI, could be engineered into the virus. A similar modification may be made if the target viral genome is VZV or another herpes virus. For VZV, neither AscI nor FseI is found in the wild-type virus.

The unique restriction site(s) are inserted into the wild-type genome using the technique of homologous recombination at any location suitable for the insertion of an exogenous DNA sequence encoding a desired protein or peptide. Methods for performing conventional homologous recombination are provided in a variety of genetic engineering texts. See, for example, Sambrook et al, *Molecular Cloning. A Laboratory Manual*. 2nd edit., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989). This procedure of the invention does not mutate the viral genome at any other location.

Once the viral genome is modified by the restriction enzyme site(s), any desired exogenous sequence, such as a universal cloning sequence or a sequence coding for a desired product, e.g., a gene, which has previously been inserted into a plasmid using conventional plasmid construction as discussed below, can be inserted into the viral genome by simple restriction enzyme cleavage and ligation at the unique restriction sites. Thus subsequent embodiments of the modified virus can be quickly made without otherwise altering the viral genome.

For example, a first DNA sequence, or cassette, which contains a selected DNA sequence encoding a selected reporter protein or peptide flanked at both ends of the sequence by the unique restriction site can be ligated into the unique restriction site of a virus modified as described above. In one embodiment, where insertion of a second foreign restriction is desired, the cassette may also contain the sequence encoding the second foreign restriction enzyme site. Once the

cassette is inserted into the virus, the modified virus, in one embodiment, permits expression of the reporter gene product utilizing a promoter native to the wild-type virus.

Alternatively, the modified virus permits expression of the reporter gene product by use of a promoter which is not normally present in that virus. The resulting modified DNA virus vector thereby contains elements capable of readily evidencing the presence or absence of the inserted reporter gene.

Further, according to this invention, the first cassette may be readily replaced with a second cassette. Once the modified virus containing the reporter gene is digested with the appropriate restriction enzymes (corresponding to either the two foreign enzyme sites or the single foreign enzyme site and the unique endogenous restriction enzyme site), the second cassette may be directionally ligated into the modified virus at the selected site. The second cassette is a DNA sequence which contains a selected DNA sequence encoding a selected protein or peptide flanked at one end of the sequence by the unique foreign restriction site and at the other end by an adjacent endogenous virus restriction site, or a second foreign restriction site which may the same or different from the first foreign restriction site.

In one embodiment, this newly modified virus, which now contains the second cassette encoding the selected protein or peptides, permits expression of the selected protein product utilizing a promoter native to the wild-type virus. Alternatively, the modified virus permits expression of the selected protein product by use of a promoter which is not normally present in that virus but is part of the second cassette.

Successful transfectants are readily identified and isolated in tissue culture by the absence of the reporter gene. For example, where the reporter gene is lacZ, the successful transfectants capable of expressing the protein product *in vitro* are identified when their growth in culture media containing X-gal does not produce a blue plaque, but a colorless plaque instead.

Excellent selection efficiencies of up to 90-100% have been obtained using these methods and components in contrast to the development of viral vectors by repeated occurrences of conventional homologous recombination techniques of the prior art, which demonstrate efficiencies of only about 10%.

One desirable embodiment of this invention provides for an expression system designed to employ a modified HSV vector and DNA cassette sequences suitable for insertion therein. The selected restriction recognition site which is foreign to wild-type HSV is, most preferably, PacI, characterized by the nucleotide recognition sequence TTAATTAA [SEQ ID NO: 1]. Other restriction sites not native to wild-type HSV may also be employed in the invention. For example, the inventors have determined that the restriction enzyme PmeI (New England Biolabs, GTTTAAAC [SEQ ID NO: 2]) has no site in the HSV-1 genome. The restriction site may be introduced into the viral genome by homologous recombination as discussed above or other conventional method.

A. The Cassette

The first cassette is constructed within a bacterial or other suitable plasmid which has been genetically engineered to contain a universal cloning sequence flanked by one or more selected unique restriction sites. Therefore, in one embodiment, a reporter gene is engineered to be bound by PmeI and/or PacI sites into the HSV-1 genome at any position. A

preferred reporter gene is the LacZ gene, which also serves as a selectable marker to accelerate the selection of recombinant virus. Thus, a first cassette useful in this invention is characterized by a high transfection frequency, the LacZ gene as reporter/selectable marker, and PmeI-PacI sites which can be inserted anywhere in the HSV-1 genome.

This first cassette is introduced into the modified HSV virus described in part B below in which the PacI (or an alternative foreign site) provides a site into which a desired cassette containing the reporter gene or any exogenous gene can be inserted.

The second cassette may be prepared substantially similarly to the first cassette, except that in place of the reporter gene is an exogenous DNA sequence encoding a protein or peptide which is desired for expression. The exogenous DNA sequence may be any gene or fragment thereof which is desired to be produced in an HSV vector. It can be a gene which encodes a therapeutically desirable protein. In an embodiment intended for therapy for the central or peripheral nervous system, more preferably, the gene encodes a protein useful when targeted to, and expressed in, the nervous system.

The selected exogenous DNA sequence is introduced into the plasmid at one of the sites in the universal cloning sequence. The plasmid containing the exogenous DNA sequence can be replicated in a suitable host cell. Any host cell useful in plasmid maintenance and replication may be employed in this method. Presently preferred host cells are bacterial cells, such as *E. coli*. Other cells used in conventional genetic engineering may also be used and readily selected by one of skill in the art.

The plasmid containing the second cassette, the sequence containing the exogenous DNA sequence flanked by the unique restriction sites, is removed from the bacterial plasmid in which the cassette is created by digestion with the appropriate restriction enzyme, e.g., PacI.

B. The Modified Virus

The modified HSV virus useful in this embodiment of the invention has been genetically engineered as described above to contain a reporter gene flanked by endonuclease enzyme restriction recognition sites which are foreign to wild-type HSV. Exemplary HSV strains include, for example, wild-type strain 17, a commonly available strain of HSV-1, which is neurovirulent when inoculated intraocularly at doses of 2 x 10^5 pfu/eye and gives mortality rates as high as 50% in Balb/c mice. In some embodiments in which it is desired to express the exogenous protein *in vivo*, it is preferable to use HSVs which contain only a single copy of the LAT gene. Another common HSV-1 strain, HFEM, has a 4.1 kb deletion within the BamH1-B restriction enzyme fragment sequence which encodes part of the long internal repeat of the LAT region of the viral genome [Y. J. Becker et al, Virology, 149:255-259 (1986)]. Since strain HFEM shows reduced pathogenicity and neurovirulence, it makes a good choice for modification according to this invention. Since there is only one copy of the otherwise diploid LAT gene, there will be only one inserted foreign restriction site. Other strains may be useful, but wild type strains with two LAT loci may have lower ligation efficiencies because of the extra foreign restriction site that will be introduced.

This will not be a problem when the gC locus is used as a site of insertion of the foreign restriction sites and reporter gene because there is only one copy of gC in the viral genome.

In one embodiment the location of the DNA sequence comprising the foreign restriction sites flanking the reporter gene is downstream of the HSV-1 LAT promoter, so that expression of the reporter gene is under the regulatory control of the HSV-1 LAT promoter. As demonstrated by the examples herein and by Ho et al and Dobson et al, cited above, this exogenous DNA sequence can be located at various distances, e.g., from about 26 to about 850 nucleotides, downstream of the LAT promoter. All or a portion of the LAT gene may be optionally removed from the HSV-1 genome when this exogenous DNA sequence is inserted.

The inventors have found that mutant HSV-1 viruses which do not express LAT RNAs can nevertheless form latent infections. Advantageously, the LAT promoter, although weak during acute infection, has been found to be well suited to expressing foreign genes during latent infection which lasts for the lifetime of an animal. This aspect of the invention is significant when the resulting modified HSV of this invention is used in gene therapy.

While the LAT promoter is exemplified herein, any suitable HSV promoter which is active in the latent period of HSV infection and in the cells could be used for *in vivo* expression. Where *in vitro* expression of the exogenous protein is desired in tissue culture, any known promoter of HSV or an exogenous promoter capable of directing expression of the protein in the virus may be employed. Examples of known promoters include the promoters for the neuronal specific enolase (NSE) and neurofilamant genes, among others. Thus, the reporter

gene and flanking foreign restriction sites can be located outside of the LAT locus in the HSV. According to this invention, the restriction endonuclease enzyme site which is foreign to wild-type HSV can alternatively be located anywhere in the HSV genome by substituting the flanking LAT sequences with the flanking sequences of the desired site of insertion.

In an embodiment of a modified HSV vector, the reporter gene is preferably lacZ. A lacZ gene driven by the LAT promoter does not express β-galactosidase in tissue culture but is only active during latency *in vivo* [D. Y. Ho et al, Proc. Natl. Acad. Sci. USA, 86:7596-7600 (1989)]. When lacZ operates as a reporter gene, because the modified HSV-1 vector containing the PacI-lacZ-PacI DNA sequence is grown in a suitable HSV substrate, plaques of the modified vector component containing lacZ appear as a blue colored plaque. See, e.g., Rixon et al, cited above. The insertion of the lacZ cassette within the foreign restriction sites, e.g., PacI, also serves as a selectable marker because the encoded bacterial enzyme in the cassette, β-galactosidase, can readily be detected histochemically using an X-gal substrate. See Example 1, in which plasmids used to make viruses containing foreign PacI sites that also express β-gal are described in detail. This accelerates the recombinant virus isolation process.

Alternatively, other suitable reporter genes and selectable markers, of which many exist in the art, such as drug resistant genes and the like, may alternatively be employed and selected for use in the construction of a modified HSV vector by one of skill in the art.

According to this method, the HSV virus vector containing the unique restriction site and first cassette is digested with the appropriate endonuclease enzyme

capable of cleaving the virus at that foreign restriction site, e.g., PacI, thereby removing the first cassette containing the reporter gene. The fragments of the digestion is mixed with the second cassette fragments and DNA ligase enzyme according to conventional methods. See, e.g., Sambrook et al, Molecular Cloning. A Laboratory Manual., Second Edition, Cold Spring Harbor Laboratory, (1989).

The ligase mixture is transfected by conventional techniques (see, Sambrook et al, cited above) into a suitable mammalian cell. Preferably, the monkey cell line CV-1 is useful for transfection, among other well known cell lines. When the virus plaques are isolated, virus plaques containing the second cassette containing the desired exogenous gene ligated into and replacing the "restriction site-lacZ-restriction site" sequence in the modified vector are white, due to loss of expression of the lacZ gene. Blue plaques indicate viruses which do not contain the second cassette.

The white plaques with the resulting HSV containing the exogenous gene under the control of the promoter are isolated from tissue culture and correct ligation confirmed by restriction digestion and Southern blotting according to conventional techniques.

Optimal conditions for *in vitro* ligation and transfection depend upon the molar ratios, and the amounts, of plasmid DNA and viral DNA used in these processes. The efficiency is determined by the proportion of white primary plaques against background blue plaques. In the hands of the inventors transfection efficiency has been obtained at between about 69 to about 100%.

This technique drastically reduces the time required to construct vectors and isolate cloned recombinant vector virus stocks, e.g., from about six

months with the prior art homologous recombination approach to about one month with the method of the invention, due to the high efficiency of the ligation/transfection procedure and the plaque color selection scheme of the invention.

III. Utility of The Invention

The components of the expression system of this invention are useful in the method for developing a modified HSV (or where desirable, another member of the herpesvirus family) containing, and capable of expressing, an exogenous gene, *in vivo* or *in vitro*. The methods and compositions of this invention are presently useful for the *in vitro* production of peptides and proteins in tissue cultures infected with the viruses of the invention.

It is also anticipated that the methods and compositions of this invention may be adapted for use in the *in vivo* production of such peptides and proteins for therapeutic use, where desirable. Still other uses of the compositions and methods of this invention are in gene transfer studies and for gene therapy in animals and man.

For example, the modified viruses, e.g., HSVs, containing exogenous genes produced by the method of the invention are useful as therapeutic reagents and possibly as diagnostic reagents. The modified HSV-1 of the invention may be constructed to contain a gene desired for delivery to a selected target cell and for treatment of a desired disease.

For diseases characterized by a gene deficiency, gene transfer could be used to bring a normal gene into affected tissues for replacement therapy, as well as to create animal models for the disease using antisense mutations.

The HSV-1 and other herpesvirus family vectors of the invention may be used for the treatment of diseases of the nervous system. For example, a modified HSV-1 virus of the invention, which contains a selected gene under the control of a LAT or other promoter which permits expression of an enzyme or other protein during the latent infectious stage of the virus can be used to deliver a gene to the central nervous system of a mammal to compensate for an inherited or acquired genetic deficiency. This method is useful for treating, among other conditions, lysosomal storage disease. See, e.g. U. S. patent application Serial No. 08/020,177, filed February 2, 1993. The present invention provides a much quicker and efficient method of introducing the desired gene into an HSV-1 virus, which can then be used in delivering a desired gene product to the central or peripheral nervous system, e.g. by peripheral, intranasal, intraocular, intracranial, or direct administration. The use of site-specific integration of DNA sequences to cause mutations or to correct defects is also possible.

Examples of other diseases of the nervous system and their related genes include: Parkinson's disease which is associated with human, rat and mouse tyrosine hydrolase genes 1, 2 and 3; Alzheimer's disease and Parkinson's disease which are associated with human, rat and mouse nerve growth factor beta subunit; Lesch-Nyan disease which is associated with human, rat and mouse hypoxanthine-guanine phosphoribosyl transferase gene; Tay-Sachs and Sandhoff's disease which is associated with human beta-hexosaminidase alpha chain gene, which together with glucocerebrosidase is associated with other lysosomal storage diseases. Human immunodeficiency virus (HIV) nef gene is associated with neurological symptoms in HIV-positive individuals.

Further, the modified HSV (and optionally other herpesvirus family) vectors of the invention may be used in cancer therapy, particularly for the destruction of tumors of the nervous system. For example, the modified HSV vectors of the invention can be modified to selectively target tumor cells of the nervous system. Examples of tumors which may be treated in this manner include, metastatic melanoma, gliomas, and neuroblastomas.

Other conditions for which treatment may be desired include amyloid polyneuropathies [prealbumin; H. Sasaki et al, Biochem. Biophys. Res. Commun., 125:636-642 (1984)], Duchenne's muscular dystrophy [uncharacterized muscle protein; A. P. Monaco et al, Nature, 323:646-650 (1987)], and for retinoblastoma [uncharacterized protein expressed in the retina and other tissues [W.-H. Lee et al, Science, 235:1394-1399 (1987); S. H. Friend et al, Nature, 323:643-646 (1986)].

Thus, the invention also provides a method of using the modified HSV vectors of the invention to treat such conditions. Preferably, the modified HSV vectors of the invention are formulated in a pharmaceutical composition. A pharmaceutical composition of the invention includes the modified HSV-1 vector containing a desired gene in a pharmaceutically acceptable carrier. Suitable carriers are well known to those of skill in the art and the present invention is not limited thereby. Generally, such a pharmaceutical composition contains between about 10^4 to about 10^7 plaque forming units (PFUs) per dose. These doses may be repeated as needed. Suitable routes of inoculation include local infection, which permits transmission to the appropriate nerve cells. For example, intranasal infection would result in transmission of the modified HSV-1 vector to the nerve cells of the olfactory bulb. Other suitable routes

including intracranial or intracerebral injections and intraocular infection are known to those of skill in the art. However, this invention is not limited to these specified routes.

Examples of diagnostic use of the vectors of this invention include an assessment of the state of nervous system development in the embryo. For example, genes which encode histological markers can be introduced into embryonic cells to determine lineage relationships during development and to elucidate neuronal pathways.

The modified HSVs containing the exogenous gene as described above can be used for a variety of methods employed in the study of neuronal cell biology. For example, a selected gene may be inserted into a modified HSV vector of the invention, and introduced into different neural cell types in culture, or *in vivo*, in order to study cell type-specific differences in processing and cellular fate. For example, genes encoding growth factors, oncogenic proteins, toxic peptides, or other physiologically important proteins, can be introduced into specific areas of the nervous system to study their effects on cell division, survival, and differentiation. Additionally, gene transfer or gene expression may be restricted to specific cells in the nervous system.

The following examples illustrate the preferred methods for preparing the vector of the invention. These examples are illustrative only and do not limit the scope of the invention.

Example 1 - Development of an efficient system for rapidly inserting foreign genes into HSV-1

This example describes the development of exemplary components of the present invention, i.e., a modified HSV-1 containing a foreign PacI site and lacZ gene and a plasmid containing foreign PacI sites flanking a universal cloning sequence. Once these two components are made, these conventional steps need not be repeated and a desired gene can be inserted into the construct using standard ligation/transfection techniques as described above.

To simplify the production of mutant viruses, an HSV strain was engineered by conventional genetic engineering techniques to insert foreign restriction enzyme sites at locations in the LAT locus at the site where a foreign gene is to be inserted. This virus is suitable for the construction of gene transfer vectors by ligation of foreign genes directly into the HSV DNA. The cloning site contains a β-gal cassette for selecting mutant plaques. By using direct ligation, (instead of homologous recombination) to insert the foreign gene, and blue/white selection according to the invention, the production and isolation of recombinant viruses is simpler and faster.

The following description of components of this invention will be understood in view of Figs. 1 through 5. CV-1 cells [ATCC CCL 70] and BHK cells [ATCC CRL 6281] were cultured in Eagle's minimum essential medium (MEM) (Gibco Laboratories, Gaithersburg, MD) supplemented with 200 μg/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 5% calf serum (Gibco Laboratories). The parental virus, HSV-1 strain HFEM, which has a 4.1 kb deletion between coordinates 0.762 to 0.789, corresponding to the HpaI DNA restriction fragment P of

the intact viral genome was described in Y. J. Becker et al, Virology, 149:255-259 (1986).

Plasmids were isolated and propagated in *E. coli* strains DH5α (BRL) or JM109 (Promega). All cloning steps and plasmid propagations were performed by standard procedures [T. Maniatis et al, "Molecular Cloning: A Laboratory Manual", in Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982)].

A) Construction of plasmid(pPBXS-4Z4) which contains the PacI sites and the HSV-1 flanking LAT sequences

The 3.9 kb XhoI-SalI fragment [Wolf et al, Nature Genetics, 1:379-384 (1992) (Fig. 1)] from the BamHI E fragment of HSV-1 [Post et al, Proc. Natl. Acad. Sci. USA, 77:4201-4205 (1980)], which contains the LAT sequence, was inserted into the XhoI site of plasmid Gem7zf [Promega Corporation] by blunt end ligation. This plasmid was digested with the restriction enzyme BstEII to create a 0.9 kb deletion and create the plasmid ΔBstEII [Wolf et al, Nature Genetics, 1:379-384 (Aug. 1992)].

A 35 bp-oligonucleotide containing the two underlined PacI sites (GTAACCTTAATTAATCTAGAGTCGAC TTAATTAAG; [SEQ ID NO: 3]) were synthesized and inserted into plasmid pΔBstEII at the BstE II site, resulting in plasmid p4.

A ICP6TATA-lacZ cassette (4.3 kb XbaI-SalI fragment), containing the lacZ gene under the control of the ICP6 promoter [Goldstein et al, J. Virol., 62:2970-2977 (1988)], was constructed from pBS-6L using a modification of the method of Goldstein et al, cited above. The cassette was isolated and inserted into p4 at the XbaI-SalI site. The resulting plasmid, pBPXZ-4Z4, contains the lacZ gene flanked by PacI restriction sites downstream of the LAT promoter. See Figure 1.

B) Engineering of PacI Containing Viral Vector by Homologous Recombination

HSV-1 recombinant viruses containing foreign PacI sites and the ICP6TATA-lacZ cassette at specific locations within the HSV-1 genome were generated. DNA from HSV-1 strain 17 [MRC Virology Institute, Glasgow, Scotland, UK] and strain HFEM were isolated using a modified version of the method described in P. F. Pignatti et al, Virology, 93:260-264 (1979) to obtain high quality DNA.

Viral DNA was isolated by a modification of Pignatti's method [P. F. Pignatti et al, Virology, 93:260-264 (1979)]. 80-90% confluent BHK cell monolayers were infected at a multiplicity of infection of 0.1. 48 hours post infection at 31°C, the virions were isolated from the tissue culture media at 14,000 g for 1 hour at 4°C by centrifugation. Virions were then digested by RNase A (100 μg/ml) at 37°C for 1 hour followed by incubation in the presence of 0.1% sodium dodecyl sulfate and 100 μg/ml of proteinase K at 37°C for about 4 to about 16 hours. Virion DNA was gently extracted with phenol, precipitated with ethanol, and resuspended in TE [10 mM Tris-Cl (pH 7.4) and 0.1 mM EDTA]. It was noted that DNA quality is a key step for the *in vitro* ligation and transfection. Even though the modified Pignatti's method can provide good quality DNA, the quality DNA was a variable. Whether or not virion DNA quality was good was estimated by two criteria: 1) whether or not virion DNA was intact as determined by field-reversal electrophoresis; and 2) checking whether or not virion DNA was transfectable as determined by $CaPO_4$ transfection.

The pBPXZ-4Z4 DNA (prepared as described in Part A of this example) and the viral DNA from the HSV-1 strain selected were cotransfected into CV-1 monkey

cells. Homologous recombination between pBPXS-4Z4 and the HSV-1 genome resulted in the recombinant viruses 17/pICP6-lacZ and HFEM/pICP6-lacZ containing the ICP6TATA-lacZ cassette at the BstEII site, 420 bp downstream of the LAT promoter, with a 897 bp deletion in the LAT region (Fig. 2). Because the recombinant vector viruses contain the lacZ gene driven by the ICP6 promoter which is expressed during the acute replication phase in tissue culture, the vector-infected plaques turn blue when X-gal substrate was added. When maximum CPE was observed, transfection lysates were harvested, replated at limiting dilutions on CV-1 cells, and plaques tested for β-gal activity. The frequency of putative recombinant progeny was 0.05%. Viruses from individual blue viral plaques from each experiment were purified by 3-4 rounds of limiting titrations and one isolate was named HFEM/ICP6-LacZ. Southern blot analysis was performed to confirm the presence of novel PacI sites together with the ICP6 promoter-LacZ cassette in the LAT locus. To further confirm the structure of the recombinant virus, PacI restriction enzyme digestion was performed and the expected electrophoresis pattern was obtained.

Fig. 3 provides a map of the HFEM genome and BamH1 B and E regions of the recombinant virus HFEM/pICP-lacZ. For southern blot analysis (not shown), wild-type HFEM and recombinant HFEM/pICP6-lacZ DNA was isolated and digested by BamHI and then hybridized with three probes. In one gel, HFEM/pICP6-lacZ had a 0.9 kb BstEII fragment deletion, thus the 0.9 kb BstEII probe could not detect any signal, while a 9 kb BamHIE band of wt HFEM was detected. In another gel, HFEM/pICP6-lacZ had the 4.3 kb ICP6 TATA-LacZ cassette insertion, thus 3.8 kb LacZ probe can detect 4.3 kb band of HFEM/pICP6-lacZ, while no signal of HFEM can be detected. In

another gel, 6.0 kb XhoI probe hybridized with 3.4 kb, 4.7 kb, 6.8 kb bands of HFEM/pICP6-lacZ, while hybridizing with 6.8 kb, 9.0 kb bands of HFEM.

C) Engineering of Recombinant HSV-1 Vector

In order to insert a non-homologous DNA fragment into the genome of HFEM/ICP6-LacZ prepared as described in Part B of this example, a plasmid, pLAT-lac2, containing the LAT promoter fused to the lacZ reporter gene was constructed. Plasmid pLZRV-B was constructed by isolating a 3.8 kb HindIII to BamHI end-filled fragment containing the LacZ gene with the SV40 poly A signal obtained from plasmid pCH110 (Pharmacia) and cloned into the EcoRV site of bluescript plasmid KS (Stratagene). The 3.8 kb XbaI-SalI fragment from pLZRV-B was inserted into the XbaI-SalI sites of p4, resulting in plasmid pLAT-lac (9.8 kb). pLAT-lac was then digested with StyI and XbaI and the 325 bp XbaI-StyI fragment was removed and the remaining 9.5 kb XbaI-StyI fragment self-ligated, resulting in pLAT-lac2. Thus, pLAT-lac2 contains a large SwaI to StyI LAT promoter element (1.2 kb) controlling the transcription of the LacZ gene. As described below in more detail, after *in vitro* ligation for 4.7 kb SwaI-PacI non-homologous DNA fragment together with SwaI-PacI digested viral vector DNA of HFEM/ICP6-LacZ, the ligation mixture was transfected into CV-1 cells to generate recombinant virus HFEM/pLAT-lacZ.

D) *In vitro* ligation and transfection

Plasmid DNAs cleaved with SwaI and PacI of part C above (pLAT-lacZ and HFEM/ICP6-lacZ) were electrophoresed on 1% agarose gels from which an appropriate fragment was isolated and purified using a gene clean II kit (Bio 101, Inc., La Jolla, CA). These fragments were ligated with SwaI-PacI digested virus DNA in the presence of 2.5% PEG8000. Then the ligation mixture was directly used for transfection by the calcium

phosphate precipitation method described by D.J. Goldstein et al, J. Virol., 62:2970-2977 (1988). Digested plasmid fragment (2 μg) and viral DNA (2 μg) were used in each transfection. From the resultant transfection, plates from each sample were incubated with Eagle's MEM media supplemented with 10% Calf serum until complete CPE was apparent. When maximal CPE was observed, the transfection mixtures were harvested and secondary infection with progeny virus at limiting dilutions was performed to determine the efficiency of production of recombinant HFEM/pLAT-lacZ virus. One day post infection, cells on each plate were overlaid with 0.5% agarose (FMC BioProducts, Rockland, ME) containing 60 μg/ml X-gal and Eagle's MEM supplemented with 10% Calf Serum.

When the vector virus contains the LacZ gene driven by the ICP6 promoter, β-gal is expressed in tissue culture (blue plaque phenotype) but it is not detectable after becoming latent *in vivo*. In contrast, a LacZ gene driven by the LAT promoter does not express β-galactosidase in tissue culture (white plaque phenotype) but is only active during latency *in vivo* [D. Y. Ho et al, Proc. Natl. Acad. Sci. USA, 86:7596-7600 (1989)]. Therefore, plaques infected with HFEM/pLAT-lacZ were white against a background of blue plaques infected with HFEM/pICP6-lacZ. After incubation for 2 days at 37°C, blue or white plaques were picked. The results are reported as the number of white plaques per total number of plaques (Table 1). In all three experiments, the yield of recombinant virus from the SwaI-PacI *in vitro* ligation and transfection was 100%.

Table 1

Efficiency of the SwaI-PacI *in vitro* Ligation and Transfection System

Experiment	Dilution[a]	No. of White plaques	No. of total plaques	%white[b] plaques
1	10^{-4}	9	9	100
2	10^{-4}	11	11	100
3	10^{-4}	21	21	100

[a] Dilution of transfected cell lysate used for white-plaque assay.

[b] Percent white-plaque value are (white plaques/total plaques) x 100.

All viruses were purified to homogeneity through three or four rounds of plaque purification and viral DNA was analyzed by restriction enzyme digestion followed by Southern hybridization. Southern blotting experiments were performed to verify the desired construction of recombinant viruses HFEM/pLAT-lacZ. For southern blotting, wild-type HFEM and recombinant HFEM/pLAT-LacZ DNA was isolated and digested by BamHI and then hybridized with two probes. In one gel, HFEM/pLAT-lacZ had 3.8 kb ICP6 LacZ cassette insertion, thus 3.8 kb LacZ probes can detect a 7.1 kb band of HFEM/pLAT-lacZ, while no signal of HFEM can be detected. In another gel, 6.0 kb XhoI probe hybridized with 4.4 kb, 6.8 kb, 7.1 kb bands of HFEM/PLAT-lacZ and also hybridized with 6.8 kb and 9.0 kb bands of HFEM.

Example 2 - Use of recombinant vector

The following example demonstrates the therapeutic use of the present invention. β-glucuronidase (GUSB), expressed in a modified HIV-1 viral vector described above, is useful for the treatment of the lysosomal storage disease mucopolysaccharidosis (MPS) VII (Sly disease).

A GUSB gene is inserted into the HSV-1 viral vector obtained as described in Example 1 using a suitable DNA restriction enzyme and standard ligation techniques. Recombinant viruses containing the GUSB gene are identified by a white plaque.

The recombinant virus vector is then used to infect MPS VII animals by corneal inoculation to target the appropriate neurons of the CNS. Briefly, the animals, e.g. mice, are anesthetized, their corneas abraded with the tip of a 30 gauge hypodermic needle and 20 μL of culture fluid containing 10^5 to 10^6 PFU of recombinant virus vector is put on each eye. The fluid is then allowed to be absorbed. The virus vector then infects the appropriate nerve cells and expresses the GUSB gene therein, thus supplying the deficient gene to the cells.

Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the methods of the present invention are believed to be encompassed in the scope of the claims appended hereto.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Wistar Institute of Anatomy and Biology

(ii) TITLE OF INVENTION: Modified DNA Virus Vectors and Uses Therefor

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Howson and Howson
(B) STREET: Spring House Corporate Cntr P.O. Box 457
(C) CITY: Spring House
(D) STATE: Pennsylvania
(E) COUNTRY: USA
(F) ZIP: 19477

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: WO
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/
(B) FILING DATE: 29-JUN-1994

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/097,965
(B) FILING DATE: 27-JUL-1993

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(A) NAME: Bak, Mary E.
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(C) REFERENCE/DOCKET NUMBER: WST44APCT

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 215-540-9200
(B) TELEFAX: 215-540-5818

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TTAATTAA 8

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTAACCTTAA TTAATCTAGA GTCGACTTAA TTAAG 35

WHAT IS CLAIMED IS:

1. A method for expressing an exogenous DNA sequence encoding a selected peptide or protein in a selected DNA virus comprising the steps of:

(a) providing a modified DNA virus produced by inserting a first unique restriction enzyme site foreign to the unmodified virus at a selected locus, said first unique enzyme site being proximal to a second unique viral restriction site; digesting said virus with enzymes which cleave at said unique restriction enzyme sites and inserting into said digestion site a first plasmid cassette which comprises a lacZ gene, said cassette flanked by two restriction enzyme sites which are the same as said first and second unique restriction enzyme sites; wherein said virus is capable of expressing said lacZ protein and wherein when grown in tissue culture in the presence of X-gal blue viral plaques are generated;

(b) digesting said modified virus with said first unique restriction enzyme and said second restriction enzyme to remove said lacZ containing cassette;

(c) inserting directionally at said digestion site a second plasmid cassette which comprises a DNA sequence encoding a selected protein or peptide flanked by said first unique restriction site and said second restriction site; wherein when said virus is grown in tissue culture in the presence of X-gal the virus produces colorless viral plaques which express the protein or peptide encoded by the sequence of said second cassette; and

(d) isolating said viral plaques capable of expressing said peptide or protein by identifying and isolating colorless plaques; and

(e) recovering the product expressed by said colorless viral plaques.

2. The method according to claim 1 wherein the second unique restriction enzyme site is endogenous to the unmodified virus.

3. The method according to claim 1 wherein the second unique restriction enzyme site is foreign to the unmodified virus.

4. The method according to claim 3 wherein the second restriction enzyme site is different from the first restriction enzyme site, thereby permitting directional insertion of the cassette into the digestion site.

5. The method according to claim 1 wherein the first cassette further comprises a promoter which controls the expression of the lacZ gene.

6. The method according to claim 1 wherein the second cassette further comprises a promoter which controls the expression of the selected protein or peptide.

7. The method according to claim 1 wherein said first unique restriction enzyme site is located downstream of a native viral promoter, and wherein insertion of the first cassette places the DNA sequence encoding said lacZ gene at a locus wherein its expression is directed by said viral promoter.

8. The method according to claim 1 wherein said unique restriction enzyme site is located downstream of a native viral promoter, and wherein insertion of the second cassette places the DNA sequence encoding said peptide or protein at a locus wherein its expression is directed by said viral promoter.

9. The method according to claims 5 through 8 wherein said promoter is selected from the group consisting of the ICP6 promoter, the LAT promoter, the actin promoter and the neurofilament promoter.

10. The method according to claim 1 wherein said virus is a member of the family Herpesviridae.

11. The method according to claim 10 wherein said virus is Herpes Simplex.

12. The method according to claim 10 wherein said unique restriction enzyme site is PacI.

13. The method according to claim 10 wherein said virus is cytomegalovirus.

14. The method according to claim 13 wherein said unique restriction enzyme site is selected from the group consisting of PacI, SwaI, and PmeI.

15. The method according to claim 10 wherein said virus is Epstein Barr virus.

16. The method according to claim 15 wherein said unique restriction enzyme site is PacI.

17. The method according to claim 10 wherein said virus is varicella zoster virus.

18. The method according to claim 17 wherein said unique restriction enzyme site is selected from the group consisting of AscI and FseI.

19. The method according to claim 1 where said expression occurs *in vitro*.

20. A modified DNA virus produced by inserting a first unique restriction enzyme site foreign to the unmodified virus at a selected locus, said unique site being proximal to a second unique viral restriction site; digesting said virus with enzymes which cleave at the unique restriction enzyme sites and inserting into said digestion site a first plasmid cassette which comprises a lacZ gene, said cassette flanked by two restriction enzyme sites which are the same as said first and second unique restriction enzyme sites; wherein said virus is capable of expressing said lacZ protein and wherein when grown in tissue culture in the presence of X-gal blue viral plaques are generated.

21. The modified virus according to claim 20 wherein the second restriction enzyme site is endogenous and unique to the unmodified virus.

22. The modified virus according to claim 20 wherein the second restriction enzyme site is foreign to the unmodified virus.

23. The modified virus according to claim 22 wherein the second restriction enzyme site is different from the first restriction enzyme site, thereby permitting directional insertion of the cassette into the digestion site.

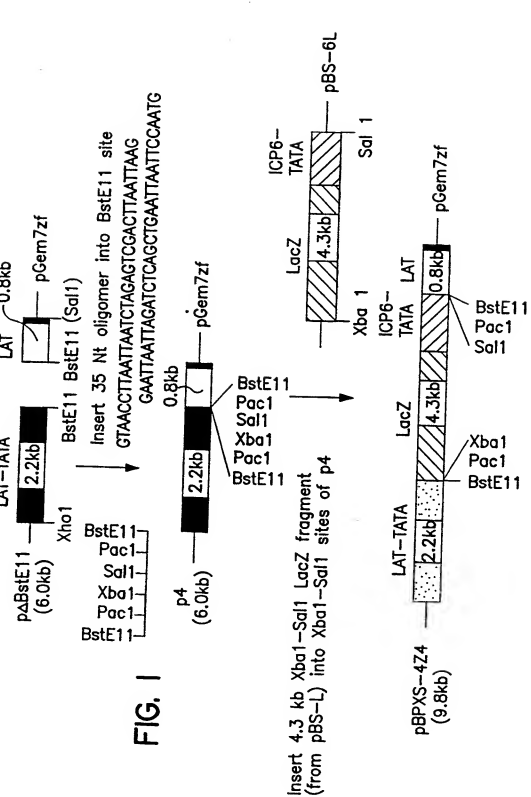
24. The modified DNA virus according to claim 20 wherein said virus is Herpes Simplex and said first unique restriction site is PacI.

25. The modified DNA virus according to claim 20 wherein said virus is cytomegalovirus and said first unique restriction enzyme site is selected from the group consisting of PacI, SwaI, and PmeI.

26. The modified DNA virus according to claim 20 wherein said virus is Epstein Barr virus and said first unique restriction enzyme site is PacI.

27. The modified DNA virus according to claim 20 wherein said virus is varicella zoster virus and said first unique restriction enzyme site is selected from the group consisting of AscI and FseI.

28. The modified DNA virus according to claim 1 wherein the first unique restriction enzyme site is located downstream of a viral promoter.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2.2kb
Xho1
pΔBstE11
(6.0kb)
BstE11
Pac1
Sal1
Xba1
Pac1
BstE11
LAT
0.8kb
pGem7zf
BstE11 BstE11 (Sal1)
Insert 35 Nt oligomer into BstE11 site
GTAACCTTAATTAATCTAGAGTCGACTTAATTAAG
GAATTAATTAGATCTCAGCTGAATTAATTCCAATG
0.8kb
pGem7zf
2.2kb
BstE11
Pac1
Sal1
Xba1
Pac1
BstE11
p4
(6.0kb)
FIG. 1
Insert 4.3 kb Xba1-Sal1 LacZ fragment
(from pBS-L) into Xba1-Sal1 sites of p4
pBS-6L
ICP6-
TATA
Sal 1
LacZ
4.3kb
Xba 1
ICP6-
TATA
LAT
0.8kb
pGem7zf
BstE11
Pac1
Sal1
LacZ
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Xba1
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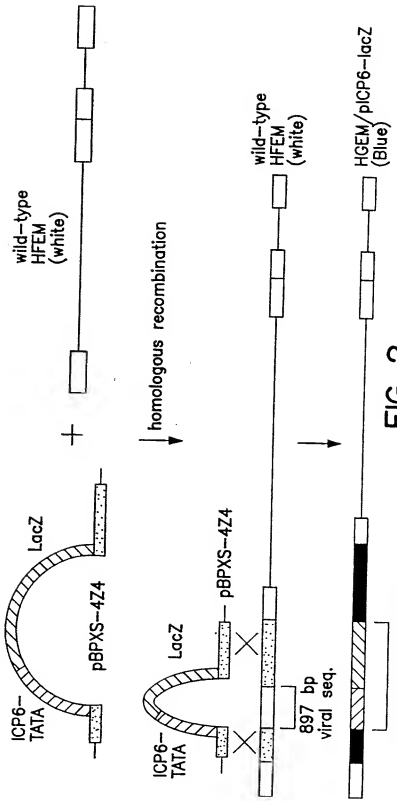
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HFEM
(white)
+
homologous recombination
wild-type
HFEM
(white)
HGEM/pICP6-lacZ
(Blue)
ICP6-
TATA
LacZ
pBPXS-4Z4
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LacZ
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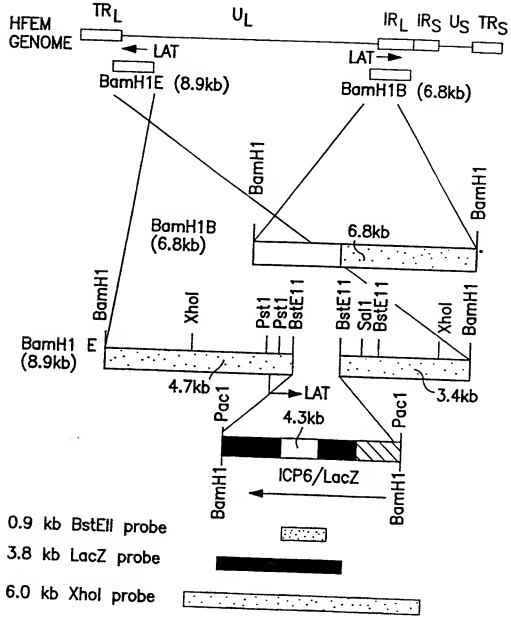
897 bp
viral seq.
ICP6 TATA-LacZ(4.3kb)

FIG. 2


HFEM GENOME
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UL
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LAT
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BamH1B (6.8kb)
BamH1
BamH1
BamH1B (6.8kb)
6.8kb
BamH1
XhoI
Pst1
Pst1
BstE11
BstE11
Sal1
BstE11
XhoI
BamH1
BamH1 E (8.9kb)
4.7kb
Pac1
LAT
3.4kb
4.3kb
Pac1
ICP6/LacZ
BamH1
BamH1
0.9 kb BstEII probe
3.8 kb LacZ probe
6.0 kb XhoI probe

FIG. 3

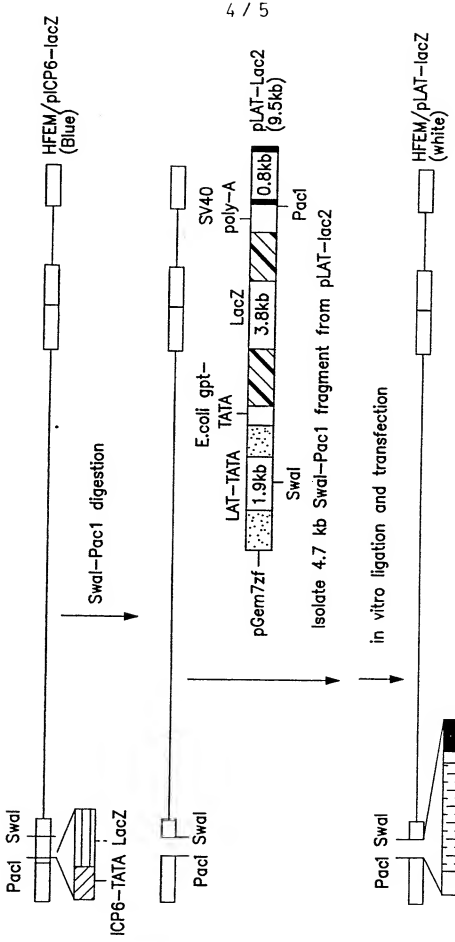
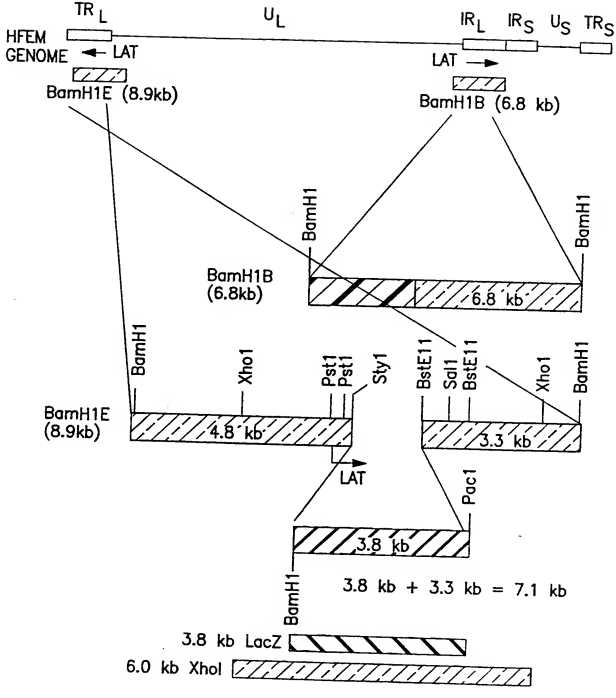

HFEM/pICP6-lacZ
(Blue)
PacI SwaI
ICP6-TATA LacZ
SwaI-PacI digestion
PacI SwaI
pGem7zf
1.9kb
LAT-TATA
E.coli gpt-
TATA
SwaI
LacZ
3.8kb
SV40
poly-A
0.8kb
PacI
pLAT-Lac2
(9.5kb)
Isolate 4.7 kb SwaI-PacI fragment from pLAT-lac2
in vitro ligation and transfection
HFEM/pLAT-lacZ
(white)
PacI SwaI
SV40-poly A LacZ LAT-TATA

FIG. 4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IR L
IR S
U S
TR S
HFEM
GENOME
LAT
LAT
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BamH1B (6.8 kb)
BamH1
BamH1
BamH1B
(6.8kb)
6.8 kb
BamH1
Xho1
Pst1
Pst1
Sty1
BstE11
Sal1
BstE11
Xho1
BamH1
BamH1E
(8.9kb)
4.8 kb
3.3 kb
LAT
Pac1
3.8 kb
BamH1
3.8 kb + 3.3 kb = 7.1 kb
3.8 kb LacZ
6.0 kb Xhol

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/08243

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12N 15/00, 15/10, 15/11, 15/12, 15/79, 15/85, 15/86

US CL :435/69.1, 172.1, 172.3, 320.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 172.1, 172.3, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog, Biosis, Medline, Biotech

Search terms: Herpesvirus, LAT, unique restriction site, expression vector

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of General Virology, Volume 71, issued 1990, Rixon et al., "Insertion of DNA Sequences at a Unique Restriction Enzyme Site Engineered for Vector Purposes into the Genome of Herpes Simplex Virus Type 1", pages 2931-2939, see Figure 1.	1-28
Y	Journal of Virology, Volume 63, Number 9, issued September 1989, Dobson et al., "Identification of the Latency-A ssociated Transcript Promoter by Expression of Rabbit Beta-Globin mRNA in Mouse Sensory Nerve Ganglia Latently Infected with a Recombinant Herpes Simplex Virus", pages 3844-3851, see page 3850.	1-28

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 SEPTEMBER 1994	26 OCT 1994

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	DAVID GUZO
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Form PCT/ISA/210 (second sheet)(July 1992)*

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, Volume 86, issued October 1989, Ho et al., "Herpes Simplex Virus Latent RNA (LAT) is Not Required for Latent Infection in the Mouse", pages 7596-7600, see Figure 1.	1-28